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TITLE: Riboswitch-Mediated Aptamer Binding for Imaging and Therapy (RABIT): A Novel Technique to Selectively Target an Intracellular Ligand Specific for Ovarian Cancer

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14. ABSTRACT
We have proposed to use a riboswitch to image or treat ovarian cancer. The riboswitch, attached to an EpCAM aptamer, will be transported into the cell where it will bind to VICKZ mRNA, a cancer cell marker, changing the conformation of the riboswitch so that it can bind to radioactive antipyrine. As a crucial first step we have selected both DNA and RNA aptamers to antipyrine and derivatives of antipyrine. We have also succeeded in showing ovarian cancer cell internalization and functional ability of a large portion of the proposed riboswitch. As an unanticipated byproduct of our work we have found that a DNA construct consisting of an EpCAM aptamer joined to an oligonucleotide complementary to VICKZ mRNA, which plays a key role in cell motility, was able to dramatically inhibit motility of ES2 ovarian cancer cells compared to untreated cells. A random DNA joined to the same EpCAM aptamer was unable to inhibit cell migration. These findings also demonstrate that we have accomplished the important task of introducing a large portion of the eventual riboswitch into the cell interior where it binds selectively to the target cancer cell marker.

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INTRODUCTION

We propose a novel technique to image and treat ovarian cancer with very high specificity, low background for imaging and low toxicity for therapy. We propose to make a riboswitch consisting of a chimeric aptamer and a sensor region that can hybridize with a specific intracellular cancer cell marker. The target for the first aptamer, EpCAM, is a surface antigen that is up-regulated in ovarian cancer cells. This surface antigen is normally recycled into the cell interior, and should carry the riboswitch with it into the cell. Once inside the cell, the riboswitch will be exposed to and interact with the intracellular mRNA cancer marker, VICKZ mRNA. This interaction with the intracellular cancer marker will change the conformation of the riboswitch to expose a second aptamer in the correct conformation to bind administered radioactive antipyrine. Antipyrine is a small molecule which rapidly enters and exits the cell interior and distributes like body water. Only cells which have both the extracellular cancer surface marker and the intracellular cancer marker will bind administered antipyrine. By radioactively labeling the antipyrine with different radioactive isotopes of iodine it will be possible to image the cancer cells (^{125}I and ^{123}I) and treat the cancer (^{131}I). In contrast to other toxins, radioactive antipyrine need not enter every cell to eradicate a tumor, since crosstalk from the radiation in one cell will also damage surrounding cells.

BODY

Tasks 1-3 – Optimization and calibration of an aptamer that binds a radioactive target

Our Statement of Work, included in the appendix to this Progress Report, describes the use of a thyroid hormone aptamer as the aptamer exposed by action of the riboswitch. This aptamer was initially selected because thyroid hormone is readily taken up by most mammalian cells, including ovarian cancer cells. It can also be readily labeled with different isotopes of iodine for imaging and therapy. In addition, an aptamer to thyroid hormone, with a description of its isolation, had already been published prior to the time of our proposal. We worked for several months using the RNA oligonucleotide described as an aptamer to thyroid hormone that contained modified nucleotides to provide RNAse resistance, but we were unable to show specific binding to either T3 or T4 thyroid hormone. However, we did show uptake of a fluorescently labeled construct containing the EpCAM aptamer joined to the reported thyroid aptamer.
Since the reported thyroid hormone aptamer was not functioning in our hands, we decided to build our riboswitch with an aptamer to an alternate target. We turned to antipyrine as a good candidate for the second aptamer in the riboswitch, which would be exposed upon riboswitch binding to VICKZ mRNA. Antipyrine can be readily labeled with isotopes of iodine, rapidly diffuses in and out of cells, and has a short biologic half-life. Since there are no published reports of an antipyrine aptamer we have been forced to isolate our own antipyrine aptamer. Because of these features of antipyrine, the development of an antipyrine aptamer appears to have a wide potential in the identification of any intracellular cell marker, including cancer cell markers, using the novel riboswitch methods we have proposed.
The Yisraeli lab began the search for an RNA aptamer to antipyrine, using modified RNA nucleotides (2′-fluoro-pyrimidines) to inhibit RNAses. A template library was synthesized consisting of fixed, adaptor sequences flanking a 33 nucleotide long sequence constructed by random incorporation of nucleotides at each position. As shown schematically below (fig. 1), in vitro transcription of these templates in the

presence of the fluoro-modified nucleotides generated a highly complex (4^{33}~10^{20}) population of modified RNAs. After subtracting out the RNAs that bind to control beads, the remaining RNAs were bound to beads linked to aminoantipyrine via carboxyl groups, washed at low salt concentrations, and then eluted either with soluble antipyrene or high salt. Recovered RNAs were reverse transcribed and the cDNAs used as new templates for transcribing RNA for the next round of binding. Three rounds of selection were performed, and samples were sent for deep sequencing from the first and third rounds of selection (both antipyrine-eluted and high salt-eluted) and from the original, non-selected library. Initial analysis of the reads obtained from the MiSeq sequencer show that while the orginal, non-selected library is highly random, with no read appearing more than 4 times (out of approximately 5 million reads), one round of selection yields a number of reads that are enriched 100 times or more. Some of these reads contain motifs that are also significantly enriched in the RNAs after the 3rd round of selection. These sequences are now being synthesized for further analysis using a Biacore Surface Plasmon Resonance (SPR) machine. In addition, the Yisraeli lab has also bound aminoantipyrine to a Biacore chip with a carboxylated surface and made use of SPR to preparatively select for those RNAs binding to the immobilized aminoantipyrine. These samples have undergone three rounds of selection as well and are currently waiting to be sequenced. The use of these complementary approaches should highlight sequences that are good candidates for highly specific antipyrine binding.

Figure 1. Schematic representation of _high throughput sequencing to identify aptamers to antipyrine

Meanwhile, to avoid the use of modified nucleotides, the Kolodny lab also began a search for DNA rather than RNA aptamers to antipyrine. A modified SELEX approach was used, followed by next generation sequencing. Aminoantipyrine was linked to carboxyl groups on side arms of superparamagnetic beads. After incubation of the antipyrine linked beads with a library of approximately 10^{14} 40 base random DNA sequences, bracketed with distinct, fixed 5' and 3' primers, the beads were extensively washed. The attached oligonucleotides were then eluted, amplified by PCR for only 15

rounds to avoid selection of only those oligonucleotides showing preferential PCR amplification, and used in a subsequent round of aptamer selection. After 5 rounds of SELEX selection the resulting sequences had adapters and index sequences added for sequencing using an Illumina next gen massively parallel sequencer at the Hebrew University in Jerusalem, Israel, the home institution of the Yisraeli lab.

Data from the sequencer was analyzed using the Genome Tools tallymer software for counting, indexing and searching k-mers, obtained on the internet from Stefan Kurtz at the Center for Bioinformatics at the University of Hamburg in Hamburg, Germany. From the Illumina sequencer we analyzed separately sequences of lengths 30, 25, 20,17,16,15,14,13,12 and 11 nucleotides. For each k-mer we searched for sequences appearing greater than 100 times. All k-mers greater than 15 nucleotides were contaminated with adaptors mid-sequence, or primers, or contained long runs of a single nucleotide. We obtained from Integrated DNA Technologies (IDT), oligonucleotides corresponding to the ten most frequently appearing sequences for each k-mer 15 nucleotides or smaller in length. The oligonucleotides were labeled with ^{32}P at the 5' end and then tested in a binding assay with aminoantipyrine joined to magnetic beads. Figure 2 shows the results of a binding assay with 12mer2, which showed a particularly low binding constant as analyzed by GraphPad Prism version 6.0 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.
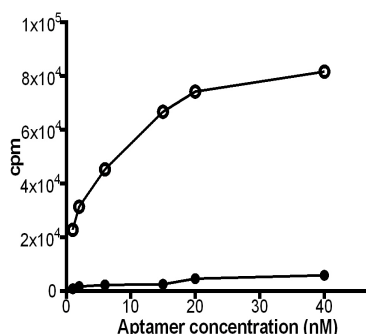
Fig. 2 Binding kinetics of antipyrine and aptamer
Radiolabeled aptamer at increasing concentrations was applied to antipyrine linked to magnetic beads. The beads were washed and radioactive decays counted. Values from three experiments were averaged, and the calculated dissociation constant (K_d) was 3.9+/-0.8 nM. Open circles represent cpm for aptamer binding to antipyrine linked to magnetic beads, while solid circles are cpm for binding of aptamer to magnetic beads without antipyrine.

Antipyrine is a small molecule (MW 188) which provides particular challenges for aptamer selection. To confirm that the sequences we had chosen were suitable aptamers, we next performed competition assays with antipyrine, aminoantipyrine and iodoantipyrine. In particular we wanted to be sure that we had selected at least one or more aptamers that would bind iodoantipyrine, since we intended to eventually use antipyrine labeled with radioactive iodine isotopes as a target for our aptamer. The antipyrine, aminoantipyrine and iodoantipyrine were combined separately to each aptamer candidate and then incubated with the bead bound antipyrine. The majority of the competition assays showed limited competition with the antipyrine bound to the beads. Because control experiments using the carboxylated beads without attached antipyrine showed very low binding of radiolabeled aptamers, it would appear that the aptamers are showing strong binding not to the antipyrine structure alone, but to the structure which encompasses both the antipyrine and its link to the arm of the magnetic bead. After obtaining the structural formula of the arm connecting the bead to the carboxyl group, we are now testing short portions of the arm bound to aminoantipyrine as the target for our aptamer candidates.

Task 4 – Testing the ability of an EpCAM aptamer to direct delivery of attached molecules into cells

The VICKZ family of proteins are associated with cell migration or movement and are abundant in embryonic cells and many cancer cells (Yisraeli, 2005). We have proposed using an RNA oligonucleotide which will hybridize to VICKZ mRNA as part of our riboswitch. When the antiVICKZ oligonucleotide binds to VICKZ mRNA it will change the conformation of the riboswitch to activate binding of the second aptamer to antipyrine. We tested the ability of an RNA aptamer recognizing EpCAM (Shigdar et al 2011) that was joined to antiVICKZ (synthesized with modified nucleotides for resistance against RNAses) to direct intracellular uptake in cultured cells. ES2 ovarian cancer cells were exposed to fluorescently labeled EpCAMaptamer/antiVICKZ and viewed by confocal microscopy. Figure 3 shows that cells exposed to the construct had increased signal compared to background fluorescence in untreated cells, indicating that the EpCAM RNA aptamer is taken into the cells during EpCAM recycling.
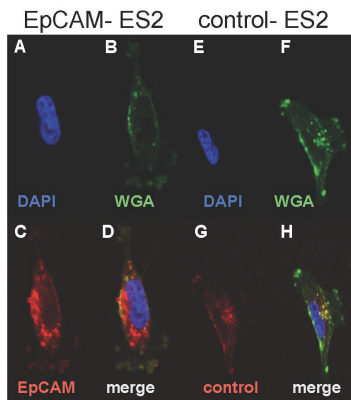
Fig. 3 Uptake of EpCAM/antiVICKZ aptamer. Fluorescently labeled construct consisting of antiVICKZ joined to EpCAM aptamer was incubated with ES2 cells. Uptake was assayed by confocal microscopy. A-D, ES2 cells incubated with 1.8uM EpCAM/antiVICKZ labeled with texas red fluorophore. E-H, ES2 cells with no construct added. DAPI (4',6-diamidino-2-phenylindole) stained nuclei are seen in blue, WGA (wheat germ agglutinin) stained cell membranes are seen in green. Most of the ES2 cells incubated with the antiVICKZ /EpCAM aptamer show increased red staining compared to control cells, indicating uptake of the red fluorescing construct.

Task 5 – Testing the ability of an EpCAM aptamer/antiVICKZ oligo to be internalized and recognize VICKZ mRNA

Since the VICKZ family of proteins are associated with cell migration or movement and are abundant in embryonic cells and many cancer cells (Yisraeli, 2005), we assessed the suitability of VICKZ as an internal cancer cell marker, by measuring the effect of a DNA oligo complementary to a portion of VICKZ mRNA, joined to a DNA aptamer of EpCAM (Song et al 2013), on the ability of ES2 ovarian cancer cells to migrate. ES2 ovarian cancer cells in culture were exposed to the EpCAM/antiVICKZ construct. The cells were then transferred in serum free media into Boyden chambers (tissue culture inserts with polyethylene terephthalate membrane of pore size 0.8um) in wells of serum-containing media. The established chemoattractive gradient caused the cells to migrate through the membrane toward the serum-containing media. On inspection and cell counting of up to 10 microscopic fields for each assay, the treated cells showed striking inhibition of migration compared to control untreated cells (figure 4). The number of treated cells showing migration through the membranes was only 3% of the number of untreated cells showing migration. As a control for nonspecific effects of DNA either on the exterior or interior of the ES2 cells we incubated the ES2 cells with a construct consisting of the EpCAM aptamer joined to a nonspecific DNA sequence. There was no statistically significant difference between the control cell migration and

the migration of cells incubated with a construct containing a nonspecific DNA sequence. These results suggest that the EpCAM aptamer binds to the EpCAM on the exterior of the cells, and that the construct is carried into the cell during EpCAM recycling. It is our assumption that once inside the cell, the antiVICKZ DNA hybridizes with VICKZ mRNA. RNAse H then degrades the mRNA portion of the mRNA-DNA hybrid, resulting in knockdown of VICKZ protein. This hypothesis is currently being further tested.
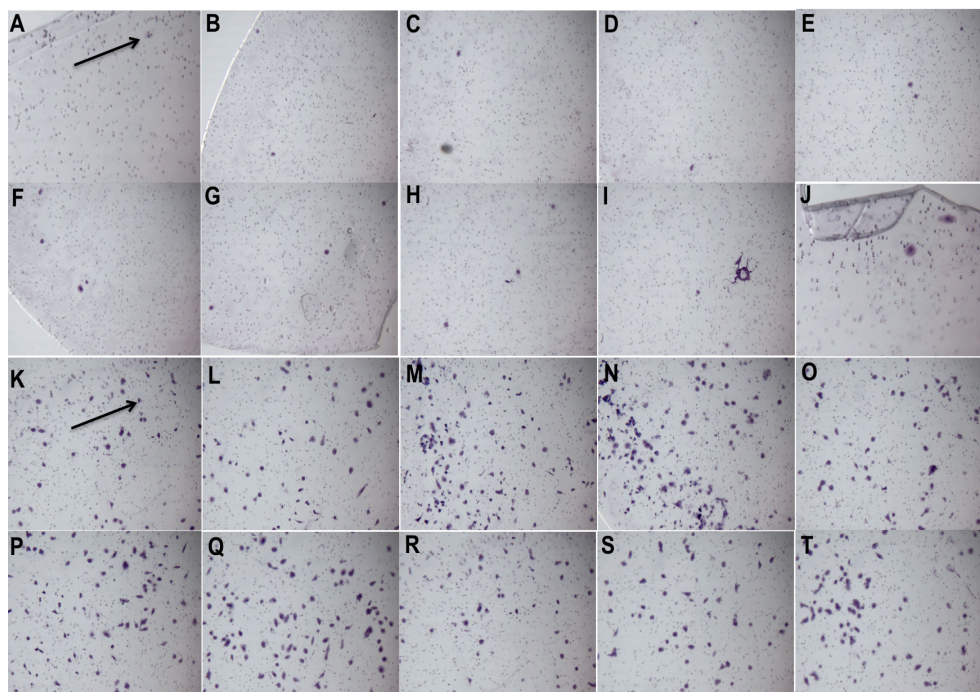

Figure 4. Migration of ES2 ovarian cancer cells. ES2 ovarian cancer cells in culture were allowed to adhere overnight to tissue culture plates and were then exposed to 3.2uM EpCAM/antiVICKZ3 (panes A-J) or no construct (panes K-T) for 2 hours. The cells were trypsinized, the trypsin neutralized with serum containing media and the cells resuspended in serum-free media and placed onto Boyden chambers in wells of serum-containing media where they incubated for 5 hours. Non-invading cells were removed and cells were fixed and stained with Toluidine Blue. Each condition was examined under 100X magnification and cells from ten random fields were counted. Arrow indicates a cell, while the smaller spots are the pores of the membrane.

Besides confirming that at least a portion of the eventual riboswitch could be taken up by the ES2 cells in culture, these results suggested to us the possibility of using the EpCAM/antiVICKZ DNA construct to limit ovarian cancer cell mobility and metastasis of treated cells. The VICKZ family of proteins includes 3 major families of proteins. Our experiments thus far have focused on only VICKZ 3. We are now exploring the use of EpCAM aptamer joined to antiVICKZ sequences for VICKZ 1 and VICKZ 2 and a combination of all three constructs together in ES2 migration assays. If these experiments continue to demonstrate decreased cell migration greater than 90% we will test inhibition of invasion through matrigel in the Boyden chamber and perform further control experiments before embarking on animal experiments.

KEY RESEARCH ACCOMPLISHMENTS

- Selection of candidate antipyrine modified RNA aptamers
- Selection of candidate antipyrine DNA aptamers
- Determination of binding constants of DNA aptamers
- Demonstration of the intracellular uptake of constructs of EpCAM and portions of the riboswitch
- Demonstration of the EpCAM aptamer/antiVICKZ oligo portion of the riboswitch to recognize and hybridize with VICKZ mRNA
- Dramatic reduction in ovarian cancer cell migration with EpCAM aptamer/antiVICKZ treatment with cells in culture.

REPORTABLE OUTCOMES

A grant application has been submitted to provide NIH funding to explore the use of riboswitch technology as we have proposed to image and treat colorectal carcinoma. An additional NIH grant application is currently being prepared which will further explore the effect of EpCAM aptamer/antiVICKZ DNA on the mobility of additional lines of cancer cells in culture and the effect on implanted ovarian cancer tumors in animals. Two manuscripts are now in preparation to describe our work on the selection of antipyrine aptamers and the effects of the EpCAM aptamer/antiVICKZ DNA on cell motility.

CONCLUSION

We have selected both DNA and RNA aptamers to antipyrine and derivatives of antipyrine. We have also succeeded in showing ovarian cancer cell internalization and functional ability of a large portion of the proposed riboswitch. After design of the folding of the riboswitch, we will be in a position to test the ability of the riboswitch to selectively bind radiolabeled targets composed of antipyrine or antipyrine derivatives. Finally this will permit us to test the ability of the cell internalized riboswitch to bind the radiolabeled targets for imaging or cell killing of ovarian cancer cells and have limited effect on other cell lines which do not contain significant amounts of VICKZ mRNA.

As an unanticipated byproduct of our work we have found that a DNA construct consisting of an EpCAM aptamer joined to an oligonucleotide complementary to VICKZ mRNA was able to dramatically inhibit motility of ovarian cancer cells compared to untreated cells. Apparently the EpCAM aptamer portion of the construct attaches to its target on the cell surface. When the EpCAM is normally recycled into the cell interior it also internalizes the attached construct. The antiVICKZ portion of the construct then hybridizes to the VICKZ mRNA, making a DNA:RNA hybrid. The RNA portion of the hybrid is then destroyed by the normal intracellular RNAse H, resulting in decreased cell motility because of the resulting diminished VICKZ protein required for cell motility. We intend to exploit this novel finding to determine whether this effect can be exploited to restrain ovarian cancer cell metastases in animal models.

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APPENDIX

Statement of Work

Task 1. Optimizing the T3 aptamer.

A number of aptamers that bind T4 have been isolated by the SELEX
process. In order to identify the aptamer most likely to be effective in the
context of the CO constructs, we will test the ability of these aptamers to
bind T3, first in vitro and then in OC cells. These experiments will be
performed by the group in Jerusalem.

1a. Double aptamer (DA) constructs will be built, consisting of an EpCam
aptamer fused to a T3/T4 aptamer. ^{32}P-labeled DA constructs, will be
synthesized with a biotin moiety at the 5' end, and the DAs will then be
tested for their ability to bind T3-sepharose columns in vitro. These
experiments should identify the shortest, effective T3 aptamer that can
effectively bind T3 in vitro. (timeframe: months 1-3)

Task 2. Calibrating I^{125}-T3 uptake into, and release from, OC cells.

In parallel to identifying the optimal T3 aptamer, the Boston group will
determine the kinetics of I^{125}-T3 uptake into ES2 cells. We will examine the
kinetics of T3 cell uptake and discharge, and the binding to normal cell nuclear
receptors, since these kinetics will determine the background to be expected
after addition of CO and radiolabeled T3.

2a. ^{125}I T3 will be added to ES2 cells. At various times, cell activity will be
determined. (timeframe, months 1-2)

2b. Once we have determined the time to reach steady state levels, the time it
takes for the I^{125}-T3 to be released from the cells will be followed. Cells will

be incubated with ^{125}I-T3 for the time determined to reach a steady state level and then they will counted at various times to determine the washout rate of the T3.(timeframe, months 3-4)

2c.. One of the sources of the background level of T3, is the binding of T3 to normal cell nuclear receptors. In our experiments it may be necessary to block these receptors by addition of blockers such as reverse T3 or tetraiodothyroacetic acid. These blockers will be added at various concentrations to ES2 cells for various times, followed by incubation with ^{125}I T3 to determine the effect of blocking the normal T3 receptors. (timeframe, months 5-6)

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Task 3. **Optimizing retention within cells of I^{125}-T3 by the EpCam/T3 DA.**
(timeframe, months 7-13)

3a. The Boston group will determine the kinetics of ES2 cellular uptake of the EpCAM/T3 DA in comparison with the non EpCAM containing A2780 cells. ^{32}P labeled DA will be introduced into the cell media and cells solubilized at various times to determine the time to steady state level of the DA. (timeframe, months 7-8)

3b. To determine the effectiveness of theEpCAM/T3 DA constructs in cells, the constructs, with an attached biotin group, will be added to the medium of ES2 cells and allowed to be taken up by the cells for the time it was shown to result in a steady state. ^{125}I-T3 will be added to the cells, and after the time required to reach 5% of steady state levels, the cells will be washed, lyzed, and incubated with strepavidin beads that will pull down the biotinylated DAs, and any associated T3. The ability to pull down ^{125}I-T3 will be compared to DAs incubated with EpCam-negative cells and DAs fused to a shuffled T3 aptamer sequence, which is incapable of binding T3. (timeframe, months 9-13)

Task 4. **Testing an EpCam-Kras-T3 CO.**

4a The Israeli group will synthesize the EpCAM-Kras-T3 CO using the published sequences that have been shown to work in cells. The molecule will be tested in vitro, comparing synthetically synthesized wild type and mutant Kras mRNAs for their ability to activate T3 binding. (timeframe, months 4-12)

4b. The EpCAM-Kras-T3 CO will then be tested by the U.S. group in OC cells that express EpCam and mutant Kras (MDAH2774). Cells will be incubated with the CO and then incubated with ^{125}I-T3, or the two agents incubated simultaneously and the kinetics of uptake of the T3 determined. (timeframe, months 14-19)

Task 5. Creating an EpCam-VICKZ-T3 CO.

The Israeli group will generate this CO in collaboration with our consultant in New York.

5a. We will first test the ability of a series of riboswitches recognizing VICKZ3 mRNA to function as beacons in OC cells. The beacons will be designed and tested by the New York consultant. The sequence most effective as a beacon in vivo will be used to synthesize the CO, along the lines of the EpCam-Kras-T3 CO described above. The Israeli group will test and calibrate the CO in vitro. (timeframe, months 13-24)

5b. The U.S. group will then test the EpCAM-VICKZ-T3 CO in ES2 cells, using ^{125}I –T3. Cells will be incubated with this CO and either simultaneously or later incubated with the radiolabeled T3 and then the radioactivity bound to the CO determined. The whole cells will be dissolved and counted. Separately cells incubated with biotin labeled CO will be lysed, incubated with strepdavidin beads and counted to determine activity bound to the CO only. (timeframe, months 20-24).